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Characterization of Chickpea Chlorotic Dwarf Virus (CpCDV) Associated with Chickpea Stunt Disease

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ABSTRACT

Background: Stunt disease is becoming the major yield limiting factors for the chickpea production and its occurrence has been reported form different states of India. The symptoms of stunt disease caused by chickpea chlorotic dwarf virus are difficult to distinguish Mastrevirus-infected plant from other disease-causing pathogens. Therefore, it's an imperative for precise detection of causal agent of the disease for development of management strategy against chickpea stunt.

Methods: Survey for the incidence of stunt disease with most characteristic symptoms of leaf reddening and yellow orange typical to Mastrevirus infection was conducted in chickpea fields. The causal agent of the stunt was characterized and described through conventional and virus-specific PCR-based diagnostic technique.

Result: The study revealed that maximum of 60% of the chickpea stunt was observed in three districts of Uttar Pradesh with an average incidence of 12.90%. The PCR amplification using CpCDV-specific primers encoding coat protein resulted in an expected amplicon size of 350 bp. The comparison of the partial coat protein sequence of virus revealed that maximum homology of 98.70% with previously identified chickpea chlorotic dwarf virus (CpCDV) strains, indicating that CpCDV associated with the chickpea stunt. Based on molecular characterization, chickpea stunt disease caused by Chickpea chlorotic dwarf virus (ssDNA), belongs to the genus Mastrevirus which is also responsible for the lentil stunt disease.

Key words: Chickpea, Disease incidence, Mastrevirus, Phylogeny, Sequencing.

Abbreviation: CpCDV- Chickpea chlorotic dwarf virus, ssDNA- Single stranded deoxyribonucleic acid.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is a self-pollinated crop belongs to the family Leguminosae, subfamily papilionoideae and is well known for its valuable source of protein, predominantly in the developing countries. Chickpea is one of the main pulse crop cultivated and consumed in India. However, India is a net-importer for most of the period due to its decreasing in area in the northern states every year. The major reasons for this decline is biotic and abiotic stresses prevalent in different parts of the country. Chickpea stunt disease is the most prevalent viral disease in most of the chickpea growing regions of India. Upto 90-100 per cent yield loss has been reported by Kaiser *et al* (1971) and 80-95% by Kotasthane *et al* (1978). Chickpea stunt name was first used by Nene and Reddy (1976) in India and the disease is caused by Chickpea chlorotic dwarf virus (CpCDV). Chickpea chlorotic dwarf virus is a monopartite, circular single-stranded DNA of the genus Mastrevirus belongs to the family Geminiviridae. It is transmitted by leaf hopper-*Orosius albicinctus* in a circulative (persistent) and non-propagative manner (Kanakala and Kuria, 2018).

There are seven dicot-infecting Mastrevirus species have been reported from Australia, the Indian subcontinent and the Middle East and one among them is Chickpea chlorotic dwarf virus (CpCDV) (Zerbini *et al*., 2017). The symptoms of the stunt disease include small sized and reduced leaves, leaf discoloration such as yellowing in *kabuli* type and reddening in *desi* type and bushy appearance of the stunted plant. The survey of chickpea carried out Horn *et al*. (1996) were surveyed and found that Chickpea chlorotic dwarf Geminivirus (CCDV) and some Luteoviruses and Mastrevirus (Kanakala *et al.,* 2013) were associated with stunt disease of chickpea.

The symptoms caused by chickpea chlorotic dwarf virus infecting dicot plant like chickpea is difficult to distinguish Mastrevirus-infected plant symptoms from plants infected by other disease causing pathogens. Therefore, it's an imperative to detect and diagnose the causal agent of the

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disease for development of management strategy to chickpea stunt. The present study was aimed to characterize and describe the causal agent of the stunt disease through conventional and virus-specific PCR-based diagnostic technique in naturally infected chickpea.

MATERIALS AND METHODS

Virus source

A roving survey was conducted in different chickpea growing areas of Kanpur dehat, Kanpur Nagar and Fatehpur districts of Uttar Pradesh during rabi (November-March) 2016-17. The information on variety, stage of the crop, percent disease incidence (PDI) and symptoms were recorded. This research was carried out from the Division of Crop Protection, ICAR-IIPR, Kanpur (Uttar Pradesh).

In each field, a plot of 4 × 4 m area was chosen and the plants showing symptoms characteristics of the stunt disease were recorded. The total number of healthy and infected plants were counted and percent disease incidence was calculated. The overall disease incidence was recorded based on visual symptoms. The per cent disease incidence (PDI) was calculated by using the formula:

$$\text{Percent Disease Incidence (PDI\%)} = \frac{\text{No. of plants infected}}{\text{Total no. of plants}} \times 100$$

Field diagnosis of stunt disease

The virus infected and healthy plants collected during survey were observed for morphological changes inside the plant system to detect the virus manifestation. Healthy and virus infected root, shoot and collar region of the plant were horizontally cut through the sterilized scalpel blade and observed xylem, phloem and cortical cells of the tissue. The morphological changes inside the plant system was recorded.

Total DNA extraction

The total DNA was extracted from the virus infected chickpea field samples by following standard DNA extraction protocols of GSure® DNA Isolation Kit (Cat. GCC1022-50R) (GCC Biotech, Calcutta, India). The DNA was amplified using Chickpea chlorotic dwarf virus coat protein (CP) specific primers designed from the CpCDV nucleotide sequences retrieved from the NCBI database using Primer 3 Plus software.

PCR amplification and sequence analysis

The total DNA extracted from virus infected chickpea leaves were used for polymerase chain reaction (PCR) amplification. Further, the polymerase chain reactions was carried out in 25 µl of reaction mixture containing DNA template (50 ng) - 2.0 µl, 10X PCR buffer-2.0 µl, 2.5mM dNTPs-2.0 µl, 2.5 Mm $MgCl_2$-1.5 µl and 2.0 µl primer (Forward primer, 5'-TTCCAGGAGGGAATCGTTGC-3' and Reverse primer 5'- CTTCACCACAAAACGGTGGC-3'), Taq DNA polymerase (1.5 U/µl) - 0.3 µl (Genei, Bangalore, India) and nuclease free water-15.2 µl. The PCR amplification was carried out in a thermal cycler (Eppendorf, India) with an initial-denaturation of 94°C for 1 min. followed by 35 cycles each consisting of denaturation at 94°C for 1 min., annealing at 58°C for 30 sec. followed by extension at 72°C for 2 min. with final extension at 72°C for 10 min. Amplified DNA fragments were electrophoresed in 1.5 per cent agarose gel and documented by Bio-Rad gel doc system. After successful confirmation of the specific amplification, the amplified product of coat protein was sequenced (Genei, Bangalore, India). Gene sequences were explored as defined below.

Similarity searches for the nucleotide (nt) sequence of coat protein gene components were performed with all the sequences available in the GenBank database using BlastN (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences showing maximum identity score with chickpea isolate (Table 1) were retrieved and aligned using the Bioedit version 7.2. The retrieved sequences used in the analysis included, 13 from chickpea, seven from lentil, one each from tomato, cotton, okra, *Vicia faba* and *Xanthium strumarium* isolates. The nucleotide sequences aligned and phylogenetic tree was constructed by CLUSTALW using the Neighbour-Joining (NJ) method.

RESULTS AND DISCUSSION

Virus source and Symptomatology

From the roving survey in different chickpea growing regions of the Kanpur nagar, Kanpur dehat and Fatehpur districts of Uttar Pradesh revealed that stunt incidence was ranged from 5-60 per cent. Initially, the infected plants appeared as yellow orange or brown discolouration of leaves with stunted growth of the plant along with reduced leaflets and leaf size. The infected leaves of desi cultivars showed reddening (Fig 1) and *Kabuli* cultivars showed chlorosis symptoms. The symptoms were observed during flowering stage. The most characteristic symptoms of stunt infected plants was phloem browning at collar region (Fig 2).

The chickpea stunt incidence of 5-10% was recorded in Akbarpur tehsil of Kanpur Dehat (Eight locations) with an average incidence of 6.37%. Maximum disease incidence of 60% was recorded in Ghatampur Tahsil of Kanpur Nagar (Five locations) with an average PDI of 22%. In Fatehpur district (Six locations), the stunt incidence was 2-33% in which maximum incidence was recorded in the Bindki block with an average incidence of 10.33%.

Field diagnosis of stunt disease

The horizontal cut portion of root, shoot and collar portion of the virus infected and healthy chickpea plant were observed for changes in internal tissue colour. The phloem region was turned to brown colour (Phloem necrosis) (Fig 2) in the collar region of the virus infected plant. However, we observed on changes in the xylem, phloem and cortical tissues of healthy chickpea.

PCR amplification and sequence analysis

The PCR amplification of CpCDV from the DNA samples of stunt infected chickpea using coat protein specific primers resulted in the expected amplicon of 350bp which confirmed the association of CpCDV with stunt disease of chickpea. The sequence analysis of the virus amplicons comprising partial coat protein gene sequence showed that sequences derived from chickpea Kanpur isolate was more than 98.7% sequence homology with other CpCDV virus isolates. The partial coat protein sequence of the virus has been submitted to NCBI under the accession number MK 893991. The CP sequence analysis and phylogenetic tree construction provides a prediction of relatedness among chickpea chlorotic dwarf virus causing stunt in chickpea. Coat protein of the virus is highly conserved region interrelated with variable bases in Mastrevirus. These properties are useful in providing molecular tool for identification of Mastrevirus associated with other legume crops. However complete dependency on CP gene may complicate identification of Mastrevirus, otherwise can be done by conserved region. Accurate diagnosis of the virus in the infected plant sample is necessary for the management and understanding the epidemiology of the stunt disease of chickpea.

Phylogenetic analysis

The phylogenetic tree was constructed by using CLUSTALW - Neighbour-Joining (NJ) method, which showed evolutionary

Table 1: Isolates of CpCDV partial coat protein gene used for phylogenetic tree analysis.

Original host	Country	Isolate /Strain	Query cover (%)	Identity (%)	GenBank Accession number
Cicer arietinum	India	Kanpur	100	100.00	MK893991.1
Lens culinaris	Pakistan	LE-2A	98	98.71	LN865159.1
Cicer arietinum	India	D320	98	98.71	JX183064.1
Lens culinaris	Pakistan	LE-1A	98	98.39	LN865158.1
Lens culinaris	Pakistan	LE-49	98	98.39	LN864703.1
Lens culinaris	Pakistan	LE-50	98	98.39	LN864702.1
Cicer arietinum	Pakistan	NIAB-C	100	97.78	HG934858.1
Cicer arietinum	India	D14	98	98.39	JX183065.1
Cicer arietinum	India	D39	98	98.39	JX183063.1
Cicer arietinum	Pakistan	clone CCDV8	98	98.39	AM900416.1
Lens culinaris	Pakistan	LE-6	98	98.06	LN865160.1
Abelmoschus esculentus	Pakistan	364	98	98.06	KT719391.1
Lens culinaris	Pakistan	LE-E	98	98.06	KM377673.1
Xanthium strumarium	Pakistan	*X.strumarium*	98	98.06	HE610413.1
Cicer arietinum	Pakistan	clone ccdv 3	98	98.06	AM850136.1
Cicer arietinum	India	Delhi	98	97.74	MH480383.1
Cicer arietinum	India	Delhi/2583	98	97.74	MG913384.1
Lens culinaris	Pakistan	LE-13	98	97.74	LN865163.1
Solanum lycopersicum	Pakistan	330Vehari	98	97.74	KP881605.1
Cicer arietinum	Pakistan	clone ccdv 6	100	97.15	AM849097.1
Vicia faba	Sudan	CpCDV_C_SD_SD79_2013	98	97.1	KM229785.1
Cicer arietinum	Sudan	CpCDV_C_SD_FR15_2008	98	97.1	KM229768.1
Gossypium hirsutum	Pakistan	501	100	96.2	KY676352.1
Cicer arietinum	Sudan	CpCDV_C_SD_SD41_2013	98	96.77	KM229784.1
Cicer arietinum	Sudan	CpCDV_C_SD_SD30_2013	98	96.77	KM229782.1



Fig 1: Chickpea stunt symptoms (a) Leaf reddening and proliferation of axillary shoot (b) Phloem browning.

relationship of the chickpea chlorotic dwarf virus Kanpur isolate (MK 893991) with other CpCDV strains (Fig 2). The isolated chickpea chlorotic dwarf virus Kanpur isolate showed maximum 98.70% nucleotide sequence homology with other CpCDV isolates from chickpea, lentil 98.71% (LN865159.1), okra 98.06% (KT719391.1), *Xanthium strumarium* 98.06% (HE610413.1) cotton 96.20% (KY676352.1), faba bean 97.10 % (KM229785.1) and tomato 97.74% (KP881605.1).

The prevalence of stunt disease was observed in all the chickpea fields surveyed in Uttar Pradesh. Similar results were observed by Horn *et al.* (1996) where stunt incidence in farmers' fields was ranged from 0 to 45% with an average of 12%. Overall, average incidence of stunt disease was 12.90% in the three districts of Uttar Pradesh. These findings are in concordance with observations made earlier by Saxena *et al* (1991). The difference in disease incidence over localities might be due to the presence or absence of inoculum in and around the field, presence of weed hosts or collateral hosts, variation in temperature and relative humidity of the locality that may have direct influence on insect vectors population and its migration, micro and macro-climate on biology and distribution of vector, *Orosius albicinctus* a main vector for field transmission of the disease.

A total of 100 virus infected plants were observed from the field samples collected in different chickpea growing areas. Similar kind of symptoms were reported by Nene *et al.* (2012). The leaf samples from desi chickpea showing leaf reddening and yellowing/chlorosis in *Kabuli* chickpea collected from the farmer's field were established with the association of chickpea chlorotic dwarf virus belongs to Mastrevirus by PCR based analysis. Our observations are resembling with the stunt symptoms listed by Kaiser and Danesh (1971) in India justifies the conclusion that they apprehended the same disease.

The phylogenetic analysis exhibited that the CpCDV Kanpur isolate was closely related to other CpCDV strains. The CpCDV Kanpur isolate has nucleotide similarity with other CpCDV isolates previously described regardless of the host plants and geo-graphical origin of the virus isolates distributed in India and neighbouring country like Pakistan and Sudan which indicating a single gene pool of common origin (Table 1). Our results strongly supported by PCR-based methods involving gene-specific primers (Nahid *et al.*, 2011, Kanakala *et al.*, 2013; Hadfield *et al.*, 2012) have been developed to detect CpCDV from infected plant tissues. The CpCDV genome organization consists of circular ssDNA, 2.5-2.7 Kb contained in a single component (monopartite). Entire genome of CpCDV consists of four open reading frames (ORFs) such as ORF V1, ORF V2, ORF C1/C2 codes for capsid protein (26.6 kDa), movement protein (10 kDa), replication initiation protein on the complementary strand (36.3 kDa), respectively (Boulton, 2002). Conventional and molecular findings of the present results highlighted the importance and reasons for the re-emerging of the stunt disease to monitor the outbreak of disease in the future. The re-emergence of stunt disease in many chickpea growing regions of India could be due to seed borne nature

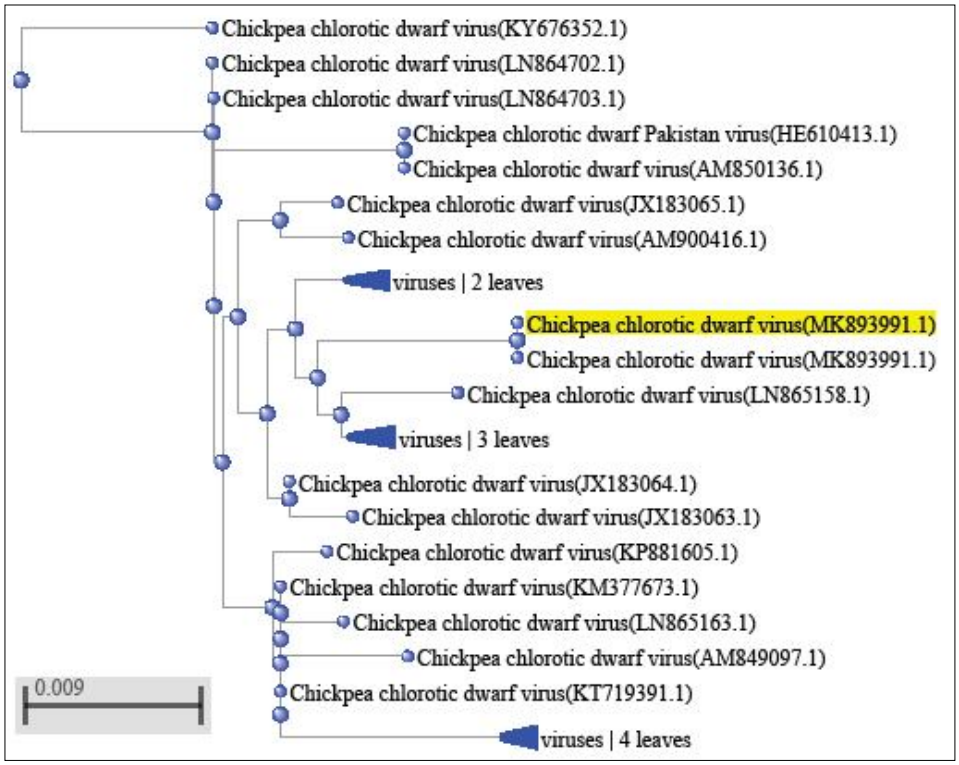

Fig 2: The phylogenetic tree constructed for Chickpea chlorotic dwarf virus showing identity with partial CP gene nucleotide in chickpea stunt disease.

of the virus, occurrence of leaf hopper vector due to climate change effect, growing of genetically narrow based chickpea cultivars, virus with wide host range for its survival are main reasons for their continuous occurrence the stunt disease.

CONCLUSION

The phloem browning in the collar region of the virus infected plant, CP gene sequence homology and phylogenetic tree with other chickpea chlorotic dwarf virus nucleotide sequences have evidently proven that chickpea stunt caused by chickpea chlorotic dwarf virus belongs to the genus Mastrevirus. Further, CpCDV is prevalent in all chickpea growing areas. The high degree of partial coat protein sequence homology among many CpCDV isolates suggest that the virus isolates so far characterized from countries like India, Pakistan and Sudan are of common origin. Therefore, continuous monitoring for the occurrence and distribution of the stunt disease needs much attention in India to avoid loss caused by the disease.

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Conflict of interest

All the authors declare that they have no competing interests. In this research does not contain any studies with animal or human topics executed by any of the authors.

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